

The PTEN/AKT Pathway In Epithelial Tumors

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The *PTEN* tumor suppressor gene encodes a protein/lipid phosphatase whose main *in vivo* substrate is phosphatidylinositol (3,4,5)-triphosphate (PIP-3), the product of PI3-kinase activity. The levels of PIP-3 are very low in quiescent cells, but rapidly increase upon stimulation by growth factors, through activation of PI3-kinase. Accumulation of PIP-3 at the membrane allows recruitment of proteins containing a pleckstrin homology (PH) domain, which binds PIP-3. Among these proteins are the Akt serine/threonine kinase family members that, upon membrane recruitment, are activated by phosphorylation. Activated AKT, in turn, phosphorylates an ever-growing list of target proteins, thus regulating key processes such as proliferation, survival, cell size and mRNA translation.



Reduced *PTEN* levels lead to increased PIP-3 and constitutive AKT activation. In fact, somatic deletions or mutations of *PTEN* have been identified in a large fraction (12-60%) of tumors, placing *PTEN* among the most commonly mutated genes in human cancer. Although loss of *PTEN* is mostly associated with advanced stage tumors, there is one notable exception, represented by the endometrioid subtype of endometrial cancer (EC).

We have focused on endometrial lesions in *Pten*^{+/-} mice as model systems to dissect the genetic and biochemical events leading to the transition from normal to hyperplastic and neoplastic epithelium. We are analyzing the natural history of these lesions and elucidating the molecular mechanisms underlying endometrial and mammary cancer pathogenesis in these mice. Specifically, we are determining if loss of *Pten* results in enhanced proliferation and/or reduced apoptosis in epithelial cells *in vivo* and *in vitro*, and whether loss of *Pten* results in enhanced response to estrogen stimulation. In addition, we want to determine whether the complete loss of *Pten* is a prerequisite for tumorigenesis or if loss of only one allele is sufficient to initiate neoplastic transformation.

Crosstalk between PTEN and ER α in endometrial cancer. Di Cristofano, Lian, Vilgelm, Beauparlant

Endometrial cancer (EC) is the fourth most frequent cancer in women, ranking first in incidence and second in mortality among female genital tract tumors. According to a widely accepted model, type I, or endometrioid, ECs are low-grade and estrogen-related, develop in pre- and perimenopausal women and are preceded by complex and atypical endometrial hyperplasia. Conversely, type II tumors are mainly papillary serous and clear cell carcinomas largely occurring in older women; they are aggressive, unrelated to estrogen stimulation, and occasionally arising in

endometrial polyps or from precancerous lesions that develop in atrophic endometrium. Loss of the 10q23 chromosomal region is a common finding in primary type I endometrial cancer, where the incidence of allelic imbalance at 10q23 is about 40%. Sequence analysis of *PTEN* has revealed mutations in about 37–51% of primary endometrioid ECs compared to less than 5% in primary type II ECs. Interestingly, the analysis of endometrial hyperplasias, which are the precancerous lesions of the endometrium, has revealed that *PTEN* is already mutated in at least 20% of cases. Thus, loss of *PTEN* is an early event in the multistep process leading to endometrioid endometrial carcinoma. We and others have

previously reported the targeted disruption of *Pten* in the mouse. Complete inactivation of the gene results in embryonic lethality. However, *Pten*^{+/-} mutants show a striking susceptibility to develop a broad array of epithelial tumors. Notably, 100% of the mutant females, in the pure 129/Sv genetic background, develop endometrial complex atypical hyperplasia by 15 weeks, and non-invasive carcinomas by 35 weeks. These lesions are dramatically accelerated by the simultaneous inactivation of *p27^{KIP1}* or *Mlh1*, further supporting the current multistep models of endometrial tumorigenesis.

As endometrioid carcinoma develops in a setting of excessive or unopposed estrogenic stimulation and loss of *PTEN* is by far the earliest and most common genetic lesion associated with type I EC, we are exploring the possibility that a functional connection exists between these two facts. Although there is increasing evidence of a crosstalk between ER α and AKT, supported for example by the demonstration that Serine 167 of ER α can be phosphorylated by AKT in breast cancer cells, *in vivo* data defining the physiological relevance of this interaction and its role in EC pathogenesis are still missing.

We have shown that loss of both (in mammary tumors) or only one (in endometrial lesions) allele of *Pten* results in the hyperphosphorylation of Akt. This feature is accompanied by the activation of a pathway that may represent a pivotal *in vivo* mechanism for neoplastic transformation consequent to *Pten* loss. In fact, both endometrial and mammary lesions show strong phosphorylation on ER α and its increased nuclear localization, perfectly correlating with the activation levels of Akt. ER α phosphorylation results, in turn, in the activation of this nuclear receptor both *in vivo* and *in vitro*, even in the absence of ligand, and in its increased ability to activate the transcription of several of its target genes. Strikingly, reduction of endometrial ER α levels and activity dramatically reduces the neoplastic effect of *Pten* loss in the endometrium, in contrast to complete estrogen depletion. Thus, we have provided for the first time *in vivo* evidence supporting the hypothesis that loss of *Pten* and subsequent Akt activation result in the activation of ER α -dependent pathways that play a pivotal role in the neoplastic process.

In summary, our data strongly suggest that supraphysiological activation of ER α is, *in vivo*,

an obligatory pathway for the development of endometrial lesions consequent to loss of *Pten* and activation of Akt. The functional connection between these molecules is further underlined by the fact that loss of PTEN is typically found in EECs expressing high levels of ER α .

***In vivo* analysis of the AKT/ER α interaction.**

Di Cristofano, Beauparlant, Brewer

Cancers originating from estrogen target tissues, such as breast and endometrium, are dependent on estrogen receptor activation for growth. We and others have shown that PI3-kinase and AKT can activate ER α *in vitro*, in tissue culture systems, even in the absence of estrogen. A specific amino acid residue, Ser167, located in the N-terminal activation function 1 (AF-1) domain of ER α , is essential for phosphorylation and activation by AKT. The *in vivo* physiological and pathophysiological relevance of AKT's role in activating ER α is still unclear. We propose that the constitutive activation of AKT through either growth factor overexpression, AKT mutation/amplification or loss of the PTEN tumor suppressor gene results in the activation of the proliferative and survival pathways regulated by ER α . To elucidate *in vivo* the relevance of ER α phosphorylation by AKT and the molecular consequences of this event, we have decided to utilize a direct genetic approach and to generate a mouse strain in which the amino acid residue targeted by AKT (Ser167) is mutated to Ala, and thus can not be phosphorylated. We have used a novel and highly efficient way to generate targeting constructs to engineer the mouse genome, called recombineering. Instead of the labor-intensive methods of cloning, this technique uses homologous recombination in highly recombinant *Escherichia coli* strains. It is our goal to use the ER α ^{S167A} knockin mouse to define in a physiological context the role of AKT/ER α crosstalk in organ homeostasis and to help identify novel pathways whose alteration contributes to neoplastic transformation in hormone-sensitive tissues.

Novel predictive indicators in breast cancer.

Di Cristofano, Brewer, in collaboration with Testa,[§] Swaby[§]

Similarly to endometrial cancer, the same AKT/ER α axis is likely to play a pivotal role in the development of ER α positive breast cancer.

Although the association between loss of PTEN and ER α expression in this tumor type is less well established, with reports describing either a direct or an inverse correlation, it has to be noted that while loss of PTEN is relatively uncommon in early breast cancer, AKT activation is extremely frequent, as a consequence, for example, of PI3KCA activating mutations or ErbB2 amplification and is often associated to ER α positive tumors. Thus, in the case of ER-positive breast cancer, AKT-mediated activation of ER α may be independent of PTEN loss. *In vitro*, this phosphorylation increases ER α transcriptional activity, even in the absence of bound ligand, and induces tamoxifen resistance. We hypothesize that this functional connection between AKT and ER α is physiologically relevant *in vivo* and may be linked to clinical outcome. To date, the frequency of the occurrence of both pAKT and (Ser167)pER α in patient samples has not been reported. To elucidate the functional relationship between activation of the PI3K/AKT pathway and phosphorylation of ER α in breast tumors, we are characterizing the prevalence and correlation of pAKT and (Ser167) pER α in a representative sample of estrogen responsive breast cancer tumor tissue samples. In addition, we are defining the correlation of coordinated pAKT and (Ser167)pER α with clinical characteristics (i.e., stage, tumor size, histologic grade, Her-2/neu status) and time to disease recurrence/progression.

***In vivo* analysis of PTEN protein phosphatase activity.** Di Cristofano, Beauparlant, Yeager

Crystallographic and *in vitro* data have shown that, in principle, PTEN might be able to act also on protein substrates. Interestingly, cells overexpressing PTEN^{G129E}, a tumor-derived mutant that lacks only the lipid phosphatase activity, also exhibited reduced cell adhesion, migration

and invasion, with the same efficiency as wild type PTEN, indicating that this phenomenon might be solely dependent on the protein phosphatase activity of PTEN. These still controversial findings suggest that these cellular functions, which alone are not sufficient to suppress tumorigenesis (the lipid phosphatase activity is in fact essential for tumor suppression), may contribute to tumor progression. To determine *in vivo* the physiological relevance of Pten protein phosphatase activity and to test the hypothesis that loss of this function is associated to neoplastic progression, we have utilized a knock-in approach to generate mice harboring the G129E mutation in the *Pten* gene (*Pten*^{+/^{PP}}).

***In vivo* analysis of Pten/Akt-dependent thyroid neoplastic transformation.** Di Cristofano, Yeager

PTEN is the susceptibility gene for Cowden syndrome, an autosomal dominant disorder characterized by multiple hamartomas and increased susceptibility to thyroid and breast cancer. Both benign and malignant thyroid disease are well-established components of Cowden syndrome. Even though somatic intragenic *PTEN* mutations are rare in uncultured primary epithelial thyroid tumors, hemizygous deletion occurs in 10–20% of thyroid adenomas and carcinomas. However, epigenetic silencing of *PTEN* and inappropriate subcellular compartmentalization are two novel mechanisms of *PTEN* inactivation associated with thyroid carcinogenesis. To better understand the pathways involved in thyroid transformation upon loss of PTEN, we have generated mutant mice with thyroid-specific inactivation of *Pten*. As expected, preliminary analysis shows extensive hyperplastic changes by three months of age. Thus, we have now a novel tool to analyze the earliest changes associated with neoplastic transformation in this organ.

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